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Genetic Manipulation for Enhancing Calcium Content in Potato Tuber

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Increased calcium (Ca) in potatoes may increase the production rate by enhancing tuber quality and storability. Additionally, increased Ca levels in important agricultural crops may help ameliorate the incidence of osteoporosis. However, the capacity to alter Ca levels in potato tubers through genetic manipulations has not been previously addressed. Here we demonstrate that potato tubers expressing the *Arabidopsis* H^+/Ca^{2+} transporter sCAX1 (N-terminal autoinhibitory domain truncated version of CAtion eXchanger 1) contain up to 3-fold more Ca than wild-type tubers. The increased Ca appears to be distributed throughout the tuber. The s*CAX1*-expressing potatoes have normally undergone the tuber/plant/tuber cycle for three generations; the trait appeared stable through successive generations. The expression of s*CAX1* does not appear to alter potato growth and development. Furthermore, increased Ca levels in s*CAX1*-expressing tubers do not appear to alter tuber morphology or yield. Given the preponderance of potato consumption worldwide, these transgenic plants may be a means of marginally increasing Ca intake levels in the population. To our knowledge, this study represents the first attempts to use biotechnology to increase the Ca content of potatoes.

KEYWORDS: ***Arabidopsis*; calcium; nutrition; potato; H^+/Ca^{2+} transporter**

INTRODUCTION

The Dietary Reference Intakes (DRI) for Ca were set at levels associated with desirable retention of body Ca to reduce osteoporosis, a condition of reduced bone density and the underlying cause of bone fragility (*1*, *2*). Consumption of adequate dietary Ca can be accomplished through a variety of diets. Diets rich in dairy products are one straightforward approach; however, intake of carbonated soft drinks now exceeds intake of fluid milk among the majority of Americans (*2*). Furthermore, some individuals and ethnic groups limit their consumption of dairy products due to lactose intolerance (*3*). After dairy products, vegetables and fruit make the next largest contribution to Ca intake and have the potential to be a significant source of dietary Ca (*4*, *5*). However, widely consumed fruits and vegetables including potato contain little Ca (*6*), so the amounts they provide constitute only minor components of the total dietary Ca intake (*5*). Given these facts, the majority of Americans do not consume enough Ca for reducing health-care problems.

Currently, an average tuber contains 20 mg of Ca, and American's eat (on average) one potato per day. Thus, there is the potential (if all potatoes were served boiled) to obtain 7300 mg of calcium per year per person from traditional potatoes (less than a weeks worth of the DRI). If we double or triple the amount of calcium in a tuber, we could provide several weeks worth of the DRI of Ca. While genetically altered foods will not "cure" osteoporosis, these transgenic plants could be a component toward boosting Ca consumption. Our goal here is not to make potatoes a superior source of Ca, but rather to improve an important staple food that is popular both nationally and internationally.

Field studies where potatoes are grown in Ca enriched soils suggest that increased Ca content may also benefit production by increasing tuber quality (*7*, *8*). However, little is currently known regarding the potential for genetic improvement of potato tuber Ca content. Ca is a required nutrient for normal plant growth and development and is especially critical to proper cell wall and cell membrane development (*9*). In potatoes, internal disorders such as hollow heart, internal brown spot (IBS), and

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brown center are the result of Ca deficiencies in the tubers (*10*). Plant scientists recommend application of Ca every two weeks during the growing season. Numerous applications of Ca during the life-cycle are probably most beneficial; however, it is difficult to "time release" the Ca supplementation throughout the growing season. Furthermore, this approach is not likely to be rigorously practiced throughout the world.

The model plant, *Arabidopsis*, contains various Ca transporters, some termed CAX for CAtion eXchangers (*11*−*13*). Several of the *CAX* genes have been cloned and characterized. The Ca levels in plants can be engineered through ectopic expression of a deregulated *Arabidopsis* Ca transporter. An *Arabidopsis* Ca antiporter (CAX1) contains an N-terminal autoinhibitory domain, and expression of N-terminal truncations of *CAX1* (s*CAX1*) in tobacco increases the Ca levels of the plants (*14*).

Here we report the generation and analysis of transgenic potatoes expressing the deregulated CAX1 transporter. Our long-term goal is to develop potatoes with enhanced Ca levels which will positively impact human nutrition and agricultural production. To our knowledge, these studies represent the first rigorous assessment of the utility of using genetic modifications to increase the Ca content in potatoes.

MATERIALS AND METHODS

Plant Material, Transformation and Growth Conditions. The potato (*Solanum tuberosum* L. var Russet Norkotah) transformation was performed via *Agrobacterium*-mediated transformation using stem explants. A 5 mm stem from potato after 6 weeks growth in vitro was excised and cultured on a MS (*15*) inorganic salts with 100 mg/L inositol, MS vitamins, 3% (w/v) sucrose, 2 mg/L zeatin, 0.3 mg/L indole acetic acid (IAA), and 0.8% (w/v) TC-agar. At the end of the one-day preculture, the stems were dipped in an *Agrobacterium* culture, blotted, and recultured on the same media for 72 h. Stem sections were then cultured on a selection medium containing MS inorganic salts, 3% (w/v) sucrose, 100 mg/L inositol, MS vitamins, 2 mg/L zeatin, 0.3 mg/L IAA, 100 mg/L kanamycin, 250 mg/L Clavamox, and 0.8% (w/v) TC-agar. Shoots developing on this medium were excised at 1−2 cm long and transferred to MS inorganic salts, 3% (w/v) sucrose, MS vitamins, 100 mg/L kanamycin, 250 mg/L Clavamox, and 0.8% (w/v) TC-agar for rooting. Cultures were maintained at 25 °C for 16 h under 60−80 μE m^{-2} s^{-1} light. After 6−8 weeks, plants which regenerated roots were transferred to the greenhouse and grown in 3 L pots filled with Metro-Mix 700 soil. Plants were regularly watered to field capacity, and once a month they were watered to saturation with 2 mM $CaCl_2$ for the first 3 months and fertilized on a weekly basis with 1 g/pot of Miracle-Gro (Scotts Miracle-Gro Products, Port Washington, NY). The temperature of the greenhouse was maintained within a range of 22 °C to 25 °C. Tubers were harvested when plants started to senesce. First-generation tubers (primary *CAX1*-expressing lines) from potatoes derived from tissue culture explants were rinsed free of soil and stored at 4 °C until shoots started to develop. These tubers of similar weight (∼45 g) were then planted and grown in 3 L pots filled with Metro-Mix 700 soil. Their successive cloned generations (second- and third-generation tubers) were produced in the greenhouse under the same growth conditions as described above.

Bacterial Strain and Plasmid. The s*CAX1* open reading frame was cloned into the *nos/nptII/nos-ter/cdc2a/nos-ter* expression vector, which was obtained from John Celenza (*16*). The plasmids, pcdc2A::sCAX1 (**Figure 1A**) and pCaMV35S::sCAX1 (**Figure 1B**) (*14*), were introduced into *Agrobacterium tumefaciens* strain LBA4404 (*17*) using the freeze−thaw method (*18*).

Southern and Northern Blot Analysis. Potato genomic DNA was extracted from leaf tissue as reported by Paterson et al. (*19*). Southern blot analysis was carried out as described previously (*20*). Total RNA was extracted from the periderm and cortical tissues of fresh potato tuber using RNeasy Plant Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Northern analysis was carried out as reported by Hirschi (*14*). DNA (5−10 μg) was digested with *Xba* I (for pCaMV35S::sCAX1) and with *Eco*R I (for pcdc2A::sCAX1) and separated in a 0.9% agarose gel by electrophoresis and blotted onto a nylon membrane (Zeta-Probe GT membrane, BIORAD Laboratories, Hercules, CA). DNA was fixed to the membrane by baking at 80 °C for 30 min. Total RNA (10 μg) was separated on 1.2% agarose gel containing 1.5% formaldehyde, blotted onto a Zeta-Probe GT membrane. RNA was fixed to the membrane by baking at 80 °C for 30 min. The probe for s*CAX1* gene was isolated from a *Not*I (1.4 kb) restriction fragment of the p039 plasmid (*14*). The membranes were prehybridized overnight at 65 °C in 7% sodium dodecyl sulfate (SDS) and 0.25 M Na_2HPO_4 and then hybridized overnight at 65 °C in the same solution containing the probe labeled with ^{32}P-dCTP using NEBlot kit (NEB BioLabs, Beverly, MA). Membranes were washed twice for 30 min each with 20 mM Na_2HPO_4 and 5% SDS at 65 °C and then washed twice again for 30 min each with 20 mM Na_2HPO_4 and 1% SDS at 65 °C. Membranes were exposed to X-ray film at −80 °C.

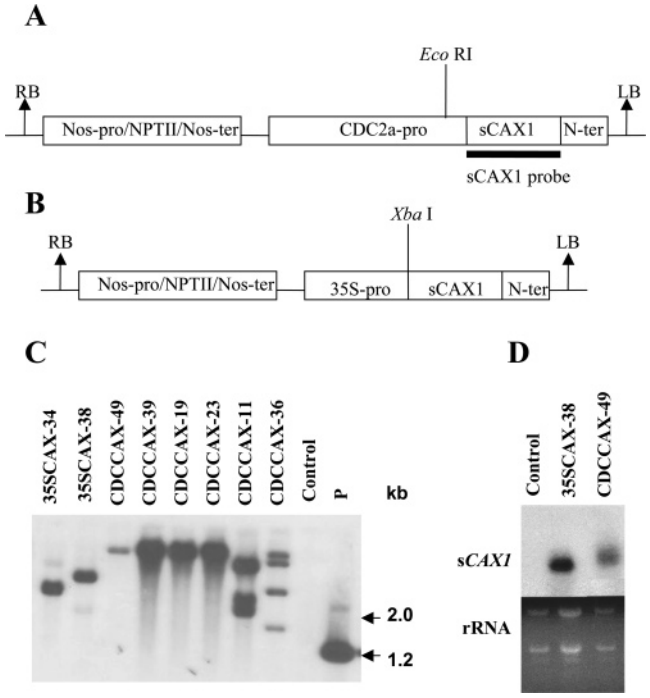

Figure 1. Molecular analyses of primary transgenic potato plants. (A–B) T-DNA regions of pcdc2A::sCAX1 (A) and pCaMV35S::sCAX1 (B). Abbreviations: RB, right border; LB, left border; Nos-pro, nopaline synthase promoter; NPTII, neomycin phosphotransferase; Nos-ter, nopaline synthase terminator; CDC2a-pro, cell division cycle promoter; sCAX1, cation exchanger 1; N-ter, nopaline synthase terminator; 35S-pro, cauliflower mosaic virus (CaMV) 35S promoter. (C) Southern blot analysis of transgenic potatoes. Five to ten micrograms of potato genomic DNA was digested with *Xba* I (for 35SCAX) and with *Eco*R I (for CDCCAX) and hybridized with the s*CAX1* cDNA probe. Lanes 35SCAX-34 and -38, transgenic potatoes with pCaMV35S::sCAX1; lanes CDCCAX-49, -39, -19, -23, -11, and -36, transgenic potatoes with pcdc2A::sCAX1; lane Control, wild-type potato; lane P, positive control of s*CAX1*. (D) Northern blot analysis of transgenic potatoes. Ten micrograms of total RNA from potato tubers was hybridized with the s*CAX1* cDNA probe. Ethidium bromide stained rRNA (bottom) is shown as a loading control.

Yield Measurements. After all greenhouse grown potato tubers were harvested and rinsed free of soil, the fresh weight of total potato tubers of each of the s*CAX1*-expressing plants controlled by CaMV 35S promoter (hereafter as 35SCAX) or cdc2a promoter (hereafter termed CDCCAX) and wild-type controls (hereafter as controls) were kept separate and obtained by the means of 16 different primary s*CAX1*-expressing lines. More precisely, 16 different primary lines (8 35SCAX lines controlled by 35S promoter and 8 CDCCAX lines controlled by cdc2a promoter) were characterized for s*CAX1* expression via Southern and/or Northern blot analysis, and the means (±SD) of 8 35SCAX

lines and the means (±SD) of 8 CDCCAX lines were compared to the means (±SD) of 6 control lines.

In successive cloned generations (second- and third-generation tubers), the fresh weight of total potato tubers from each of 4 35SCAX lines, 4 CDCCAX lines, and 4 control lines were used for yield measurements.

Calcium Analysis of the First- and Second-Generation Tubers. Harvested potato tubers and leaves were dried at 70 °C for 4 days, and a total of 0.25 g (dry weight) from each of the potato tubers and leaves was digested as reported by Feagley et al. (*21*). Total Ca content per gram of dry weight was determined by inductively coupled plasma emission spectrophotometer (Spectro, Kleve, Germany). For the primary transgenic plants, 5 tubers of similar weight (~45 g) from each of these 16 transgenic lines and 4 controls were harvested and dried, and then the Ca analysis with whole tuber tissues was carried out on individual tubers. In the second-generation plants, 5 tubers of similar weight (~45 g) from each second-generation line derived from 4 primary potato tubers (CDCCAX-36, -49, 35SCAX-34, and -38) were harvested and analyzed as described above.

Calcium Distribution Analysis of the Third-Generation Tubers. Five third-generation tubers of similar weight (~45 g) from each of the third-generation lines derived from four second-generation potato tubers (CDCCAX-36, -49, 35SCAX-34, and -38) were harvested and bisected longitudinally. Three tissue types, (1) the periderm and cortical tissue region (O), (2) the central pith region (I), and (3) the middle region between the cortical tissue region and the central pith region (M) of the tubers were sampled and dried, and then the Ca distribution analysis was carried out on individual tubers.

Statistical Analysis of the Second- and Third-Generation Tubers. All data were analyzed as a randomized complete block using SAS procedure: GLM (version 8.01, SAS Institute Inc., Cary, NC). Means were separated by Duncan's Multiple Range test (*22*) (at the 0.05 probability level) on a comparison-wise basis. Data are expressed as the means ± standard deviation (SD) of five, single-tuber replications per line.

RESULTS

s*CAX1* Expression in Potato. Ectopic expression of the N-terminal truncated version of the *Arabidopsis* CAX1 transporter, in conjunction with the biochemical properties of the CAX1 transporter in yeast, cause increased Ca levels in tobacco (*14*). However, given the Ca deficiency-like symptoms caused by s*CAX1* expression using the cauliflower mosaic virus (CaMV) 35S promoter (*14*), we opted to also express s*CAX1* under the control of the cell division cycle (cdc2a) promoter (*16*). In *Arabidopsis*, cdc2a transcript levels are correlated with the competence to divide (*16*); however, the expression of this *Arabidopsis* promoter has not been detailed in potato. In our preliminary analysis, we found that s*CAX1*-expressing tobacco plants expressed in front of the cdc2a promoter (CDCCAX) expressed less than half the amount of s*CAX1* RNA as compared to s*CAX1*-expressing plants driven by the conventional CaMV 35S promoter (35SCAX) (data not shown). In potatoes, we have generated 26 35SCAX- expressing lines and 24 CDCCAX-expressing lines.

We randomly selected and confirmed 16 independent transgenic potato lines by Southern blot analysis. Genomic DNA was digested with *Eco*R I (for pcdc2A::sCAX1) and with *Xba* I (for pCaMV35S::sCAX1) (yielding border fragments which include a portion of the inserted T-DNA and genomic DNA) and hybridized with the s*CAX1* probe. Digestion of the potato genomic DNA with these restriction enzymes revealed the transgene copy number from the number of hybridizing bands and independent transformation events from the hybridization patterns. As demonstrated in **Figure 1C** (parts of the data are shown), 35SCAX and CDCCAX lines contain various copy numbers of the s*CAX1* expression vector. The lines we have termed 35SCAX-34, 35SCAX-38, CDCCAX-49, CDCCAX-39, CDCCAX-19, and CDCCAX-23 appear to contain single insertions (Note: the independent transformants, CDCCAX-39, -19, and -23, were not able to detect different hybridization band patterns by Southern analysis due to high molecular weight fragments of larger than 10 kb). RNA gel blots documented that s*CAX1* transcripts accumulated in all of the transgenic lines (**Figure 1D**, parts of the data are shown). The inability to detect a s*CAX1* homologue in the wild-type (control) lines by Southern or Northern analysis may be due to the stringency of hybridization used in this study. Four primary transgenic lines showing single-copy insertions (35SCAX-34, 35SCAX-38, and CDCCAX-49) and multiple-copy insertion (CDCCAX-36) from Southern analysis (**Figure 1C**) were selected and subjected to further analysis of Ca accumulation in s*CAX1*-expressing second- and third-generation tubers.

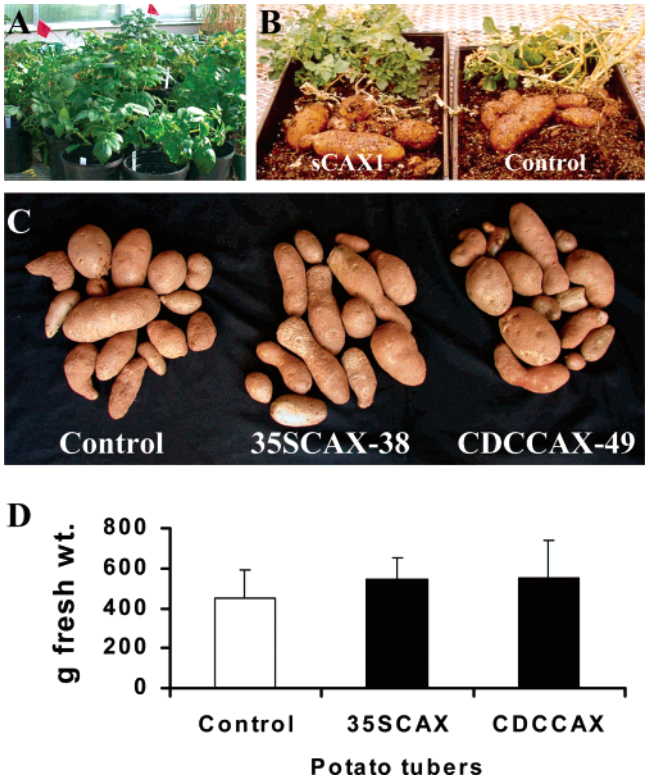

Figure 2. Phenotype of transgenic s*CAX1*-expressing primary potatoes. (A–B) The morphology and growth characteristics of the s*CAX1*-expressing the edible portion of the potatoes are indistinguishable from that of wild-type controls. Most of the plants in **Figure 2A** show the s*CAX1*-expressing plants, and red flags indicate control plants. (C–D) The yield and size distribution of edible potato tubers from s*CAX1*-expressing plants. The size distribution (C) and yield (D) of the s*CAX1*-expressing potatoes and wild-type controls. Data are presented as the means ±SD of measurements on fresh weight of total potato tubers from each of 8 35SCAX, 8 CDCCAX, and 6 control lines.

Phenotypes of s*CAX1*-Expressing Potatoes. In both the 35SCAX- and CDCCAX-expressing plants, deregulated s*CAX1* expression did not alter the morphology or growth characteristics or the edible portion of the tubers (**Figure 2A,B**). Occasionally, s*CAX1*-expressing potatoes demonstrated some weak necrotic lesions on the primary transformants grown on artificial medium and soil (data not shown). However, this phenotype was not nearly as severe as the tobacco s*CAX1*-expressing lines (*14*) and was apparent in less than 10% of the *CAX1* expressing transformants. The nectrotic lesions appeared to be evenly distributed between the 35SCAX and CDCCAX lines. Total yield (as measured by fresh weight of the potato tubers) of the primary s*CAX1*-expressing lines was indistinguishable from that

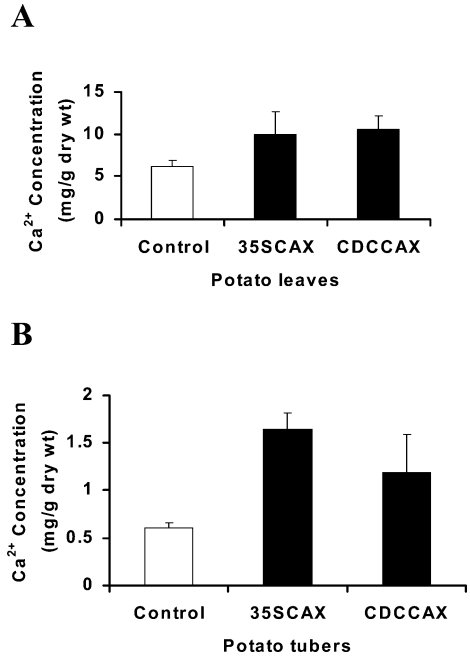

Figure 3. Ca accumulation in transgenic s*CAX1*-expressing primary potato leaves and tubers. Total Ca content of potato leaves (A) and tubers (B) was determined by inductively coupled plasma emission spectrophotometer. Data are presented as means ±SD of 8 35SCAX, 8 CDCCAX and 4 control lines (five individual replications per line).

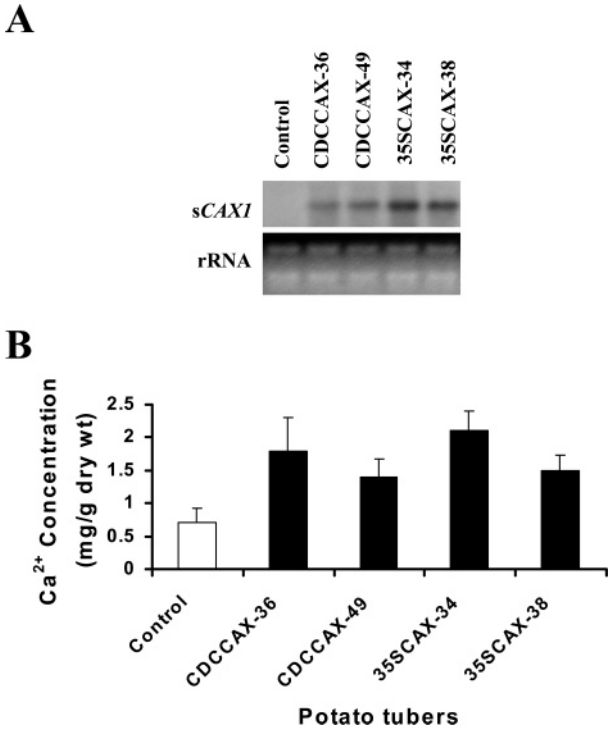

Figure 4. Northern blot analysis and Ca accumulation in transgenic s*CAX1*-expressing second-generation potato tubers. (A) Northern blot analysis of transgenic potatoes. Ten micrograms of total RNA from potato tubers was hybridized with the s*CAX1* cDNA probe. Lane control, wild-type potato; lanes CDCCAX-36 and -49, transgenic potatoes with pcdc2A:: sCAX1; lanes 35SCAX-34 and -38, transgenic potatoes with pCaMV35S:: sCAX1. Ethidium bromide stained rRNA (bottom) is shown as a loading control. (B) Ca accumulation in s*CAX1*-expressing second-generation potato tubers. Data are presented as the means ±SD of five, single-tuber replications per line.

of controls (**Figure 2C,D**). The variation in yield within the s*CAX1*-expressing lines was not significantly different from the control lines (data not shown). Furthermore, there was no correlation between Ca content within the s*CAX1*-expressing lines and yield (data not shown). The yield of all of the second- and third- generation s*CAX1*-lines also did not differ from the control (data not shown).

Calcium Accumulation in s*CAX1*-Expressing First-Generation Potato Plants. Total accumulation of Ca and other ions was measured in the edible (tubers) and aerial (leaves) portions of the potatoes. There was variability in the Ca content of the primary transgenic potatoes; however, most of the s*CAX1*-expressing potatoes contained significantly more Ca (1.5- to 3.0-fold in tubers and 1.2- to 1.7-fold in leaves) than controls (**Figure 3**). We were also interested in determining if s*CAX1*-expressing potatoes also demonstrated increased content of other minerals. No significant increase of other minerals (Cu^{2+}, Fe^{2+}, Mn^{2+}, and Zn^{2+}) was observed with any of the lines analyzed (data not shown).

Stable High Calcium Accumulation in s*CAX1*-Expressing Second-Generation Potato Plants. To confirm that the increased Ca accumulation was consistent in the next generation tubers, second-generation tubers derived from the first-generation potato tubers (CDCCAX-36, -49, 35SCAX-34, and -38) were tested. Initially, RNA gel blot analysis confirmed that s*CAX1* transcripts accumulated in all of the second-generation tubers (**Figure 4A**). Total accumulation of Ca and other ions was measured in the edible portions of the potato tubers. All of the s*CAX1*-expressing second-generation potato tubers contained 2- to 3-fold more Ca than controls (**Figure 4B**). No significant increase of other minerals (Cu^{2+}, Fe^{2+}, Mn^{2+}, and Zn^{2+}) was observed with any of the second-generation lines analyzed (data not shown).

Calcium Distribution Analysis of the Third-Generation Tubers. To analyze Ca distribution in s*CAX1*-expressing potato tubers, we bisected the tubers longitudinally and divided tubers into three tissue types, (1) the periderm and cortical tissue region (O), (2) the central pith region (I), and (3) the middle region between the cortical tissue region and the central pith region (M) (**Figure 5A**). All of the s*CAX1*-expressing third-generation potato tubers contained 2-fold more total Ca in all three tissue types when compared to controls (**Figure 5B**). Thus, the increased Ca appeared to be distributed throughout the tuber.

DISCUSSION

During the past decade, potato production in developing countries has entered a growth phase (*23*). In the United States, potatoes are the most economically significant crop in the produce industry, earning farmers nearly $2.7 billion in 2000. Ca deficient potatoes are more susceptible to many secondary infections and storage diseases (*8*, *24*). Ca is essential for formation of cell walls and cell membranes. Additionally, Ca deficient potatoes may have reduced storage life and poor quality (*9*). Transgenic potatoes sequestering more Ca thus may be more robust than traditional varieties. To potentially increase the nutritional content and yield of potatoes, we have attempted to alter the Ca content of tubers through genetic engineering. One approach to alter the Ca content in plants is to directly engineer high expression of Ca transporters in the edible portion of the plant. Simplistically, this strategy can be thought of as nutrient 'mining', where the nutrient is transported from the soil into the edible portions of the plant. Specifically, one potential model for increasing the Ca content in edible foods would be to manipulate plant endomembrane transporters to transport more Ca. In animal cells and yeast, capacitative calcium entry (CCE) mechanisms become activated when vacuolar Ca transporters

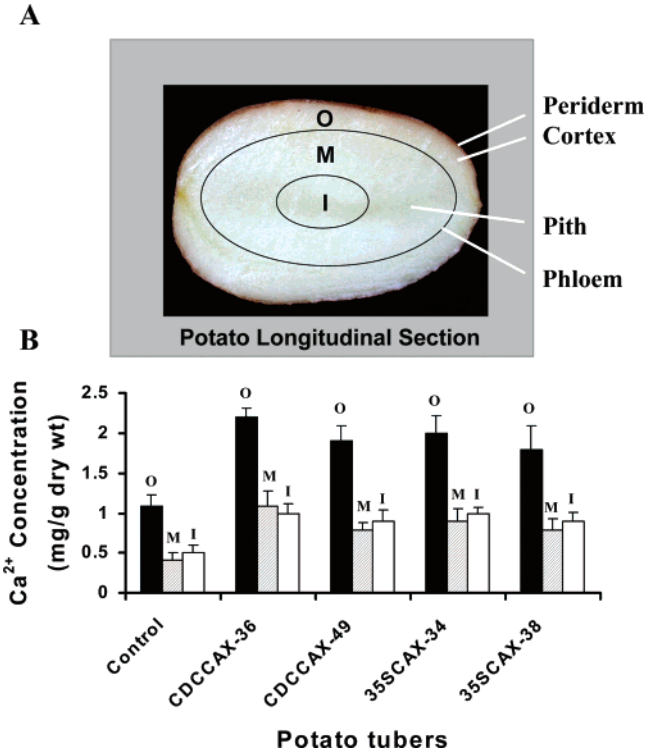

Figure 5. Ca distribution analysis of the third-generation tubers. (A) Potato longitudinal section. The tubers were bisected longitudinally and divided tubers into three tissue types; the periderm and cortical tissue region (O), the central pith region (I), and the middle region between the cortical tissue region and the central pith region (M). (B) Ca accumulation s*CAX1*-expressing third-generation potato tubers in all three tissue types. Data are presented as means ±SD of five, single-tuber replications per line.

are highly expressed (*11*). This CCE response requires putative plasma membrane voltage-gated Ca channels. In agreement with the CCE models in yeast, transgenic tobacco expressing high levels of s*CAX1* display dramatic increases in Ca content when compared to vector controls (*14*). Therefore, our working hypothesis was that increased activity of a plant Ca transporter would increase total Ca levels. Here we have confirmed our hypothesis by demonstrating that ectopic expression of a deregulated *Arabidopsis* H^+/Ca^{2+} transporter, sCAX1 (N-terminal autoinhibitory domain truncated version of CAX1), increases the Ca content in potato.

The control of s*CAX1* expression is crucial for proper modulation of Ca levels in the tuber. Promoters control the level of gene expression and where a particular gene is expressed. In plant studies, the CaMV 35S promoter is often used to give high level expression of a given gene throughout the entire plant. In previous studies using tobacco, expression of s*CAX1* driven by the CaMV 35S promoter has been shown to dramatically increase the total Ca accumulation in the entire plant, but also causes tobacco plants some alterations in growth and altered morphology, presumably through high Ca sequestration into the vacuole (*14*). However, expression of deregulated s*CAX1* in potatoes does not appear to alter plant growth (**Figure 2A,B**). We have expressed s*CAX1* under the control of the CaMV 35S promoter as well as the cdc2a promoter (*16*), and we have obtained multiple s*CAX1*-expressing potato lines expressing various copies of the transgene (**Figure 1**). The vast majority of these plants are healthy and indistinguishable from wild-type controls in their growth characteristics (**Figure 2**). These plants display up to 3-fold more Ca, and most importantly, this Ca increase appears in successive generations (**Figures 3−5**). Furthermore, the increased Ca content appears to be distributed throughout the tuber (**Figure 5**). It is interesting to speculate that s*CAX1* expression might be most productive in plants that produce edible tubers or roots. In these plants, the Ca does not need to be translocated up the entire length of the plant to achieve the desired result. However, the lack of severe phenotypes associated with the s*CAX1*-expressing potatoes may simply be a result of low level activity of the transporter or of more selective Ca accumulation in potato tubers than in aerial portions of the potatoes. In fact, the aerial portion of the potatoes accumulated approximately half the amount of Ca as compared to the aerial portion of the tobacco plants (data not shown). Moreover, exogenous Ca supplementation in the soil with 2 mM $CaCl_2$ for the first 3 months might also be a result of the lack of severe phenotypes associated with the s*CAX1*-expressing potatoes. Future work will have to be done to determine the level of sCAX1 activity in these potatoes and the extent to which vacuolar Ca^{2+} transport has been altered.

There are well-known relationship between divalent cation concentrations in plants such that increasing the levels of one can compete for divalent cation-binding sites, thus reducing uptake of the amount of other minerals (*25*). Atlternatively, we have shown in previous work that the CAX transporters can move several different cations. This study suggests no significant change in other minerals with any of the potato lines analyzed (data not shown). However, given the emerging data regarding the various biochemical properties of CAX transporters (*26*), the s*CAX1*-expressing plants will need to be grown and analyzed in a variety of growth conditions.

Ca movement is very limited in plants (*25*). For the most part, Ca is passively absorbed by roots along with water from the soil and moves passively through the plant (*27*−*29*). Since this transpiration stream is almost entirely upward to the leaves, spraying Ca onto foliage is only likely to increase the Ca content in those parts of the plant already adequately supplied (*30*). Therefore, this approach is dubious if one is trying to increase the Ca supply to the tubers. Previous studies have shown that foliage applied Ca nitrate did not increase tuber Ca (http://www.teagasc.ie/publications/pot2000/paper3.htm). Furthermore, Ca applied to foliage in early July could not be detected in new foliage at the end of July. In other words, Ca moved neither down to the tubers nor up to new foliage. This gives an indication of just how immobile Ca is in the plant. Furthermore, many soils contain Ca, but much of it is insoluble and unavailable to potatoes. This often leads to localized Ca deficiencies within the tuber. These deficiencies are associated with internal brown spot (IBS), brown center, and hollow heart of potatoes (*10*).

Some studies have demonstrated a direct link between applications of Ca to the soils and tuber quality (*7*, *8*, *24*). Adding Ca at tuber initiation virtually eliminated the incidence of IBS (*31*). However, it appears unlikely that spreading Ca salts postplanting will increase tuber Ca and decrease disease. The Ca ion can become attached to clay particles in the soil (*25*). It is therefore likely that Ca applied postplanting will be effectively locked-up close to the soil surface, with very little being available to the crop in the year of application.

As we detail in the Introduction, in terms of human nutrition, no single food source will rectify Ca intake deficiencies. The inherently low levels of Ca found in tubers will never make the s*CAX1*-expressing potatoes a panacea for deficiencies in Ca consumption. Rather, the s*CAX1*-expressing potatoes will provide some additional Ca and will be *a prototype* for the ability to alter the Ca content in many more food crops.

This study represents a unique and straightforward approach with obvious applications. To our knowledge, an appropriate molecular tool to enhance levels of Ca in potato tubers has not

been used. We have analyzed how ectopic expression of an *Arabidopsis* Ca^{2+} transporter s*CAX1*-expressing in potato plants driven by the cdc2a promoter as well as the CaMV 35S promoter consistently increases Ca content. Plants vary greatly in the amount of bioavailable Ca, meaning the amount of Ca that can be digested, absorbed, and metabolized (*25*). However, to assess bioavailability the potato tubers should be labeled with $^{45}Ca^{2+}$ or stable isotopes of Ca^{2+}, and animal feeding studies should be conducted. In previous studies with s*CAX1*-expressing tomatoes, sCAX1 expression does not appear to alter either the ethylene levels or the sugar content of the tomatoes (Park et al., unpublished data). These findings suggest that s*CAX1*-expressing potatoes taste like wild-type potatoes. However, future work will need to be undertaken to determine if the increases in Ca in the s*CAX1*-expressing potatoes might alter the sugar content, vitamin C content, glycoalkaloid level, or texture of the potatoes. Future studies will also be done to determine if these changes decrease the incidence of pathogen infection and postharvest decay, two major problems worldwide. Packaged together, these benefits could positively impact both production and the well being of consumers.

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LITERATURE CITED

(1) Bachrach, L. K. Acquisition of optimal bone mass in childhood and adolescence. *Trends Endocrin. Met.* **2001**, *12*, 22−28.

(2) Bryant, R. J.; Cadogan, J.; Weaver, C. M. The new dietary reference intakes for calcium: Implications for osteoporosis. *J. Am. Coll. Nutr.* **1999**, *18*, 406S−412S.

(3) Pribila, B. A.; Hertzler, S. R.; Martin, B. R.; Weaver, C. M.; Savaiano, D. A. Improved lactose digestion and intolerance among African-American adolescent girls fed a dairy-rich diet. *J. Am. Diet. Assoc.* **2000**, *100*, 524−528.

(4) Weaver, C. M.; Plawecki, K. L. Dietary calcium: Adequacy of a vegetariandiet. *Am. J. Clin. Nutr.* **1994**, *59*, 1238S−1241S.

(5) Weaver, C. M.; Proulx, W. R.; Heaney, R. Choices for achieving adequate dietary calcium with a vegetarian diet. *Am. J. Clin. Nutr.* **1999**, *70*, 543S−548S.

(6) Fleming, K. H.; Heimback, J. T. Consumption of calcium in the US: food sources and intake levels. *J. Nutr.* **1994**, *124*, 1426S−1430S.

(7) Kratzke, M. G.; Palta, J. P. Calcium accumulation in potato tubers: role of the basal roots. *HortScience* **1986**, *21*, 1022−1024.

(8) Olsen, N. L.; Hiller, L. K.; Mikitzel, L. J. The dependence of internal brown spot development upon calcium fertility in potato tubers. *Potato Res.* **1996**, *39*, 165−178.

(9) Kirby, E. A.; Pilbeam, D. J. Calcium as a plant nutrient. *Plant Cell Environ.* **1984**, *7*, 397−405.

(10) Clough, G. H. Potato Tuber Yield, Mineral Concentration, and Quality after Calcium Fertilization. *J. Am. Soc. Hortic. Sci.* **1994**, *119*, 175−179.

(11) Hirschi, K. D. Vacuolar H^+/Ca^{2+} transport: Who's directing the traffic?. *Trends Plant Sci.* **2001**, *6*, 100−104.

(12) Pittman, J. K.; Hirschi, K. D. Regulation of CAX1, an Arabidopsis Ca^{2+}/H^+ antiporter identification of An N-terminal autoinhibitory domain. *Plant Physiol.* **2001**, *127*, 1020−1029.

(13) Pittman, J. K.; Shigaki, T.; Cheng, N.-H.; Hirschi, K. D. Mechanism of N-terminal autoinhibition in the *Arabidopsis* Ca^{2+}/H^+ antiporter CAX1. *J. Biol. Chem.* **2002**, *277*, 26452−26459.

(14) Hirschi, K. D. Expression of *Arabidopsis CAX1* in Tobacco: Altered Calcium Homeostasis and Increased Stress Sensitivity. *Plant Cell* **1999**, *11*, 2113−2122.

(15) Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* **1962**, *15*, 473−497.

(16) Doerner, P.; Jorgensen, J.-E.; You, R.; Steppuhn, J.; Lamb, C. Control of root growth and development by cyclin expression. *Nature* **1996**, *380*, 520−523.

(17) Hoekema, A.; Hirsch, P. R.; Hooykaas, P. J. J.; Schillperoort, R. A. A binary plant vector strategy based on separation of *vir*- and T-region of the Agrobacterium tumefaciens Ti-plasmid. *Nature* **1983**, *303*, 179−180.

(18) Holsters, M.; De Waele, D.; Depicker, A.; Messens, E.; Van Montagu, M.; Schell, J. Transfection and transformation of *A. tumefaciens*. *Mol. Gen. Genet.* **1978**, *163*, 181−187.

(19) Paterson, A. H.; Brubaker, C. L.; Wendel, J. F. A rapid method for extraction of cotton (*Gossypium* spp.) genomic DNA suitable for RFLP or PCR analysis. *Plant Mol. Biol. Rep.* **1993**, *11*, 122−127.

(20) Park, S. H.; Pinson, S. R. M.; Smith, R. H. T-DNA integration into genomic DNA of rice following *Agrobacterium* inoculation of isolated shoot apices. *Plant Mol. Biol.* **1996**, *32*, 1135−1148.

(21) Feagley, S. E.; Valdez, M. S.; Hudnall, W. H. Papermill sludge, phosphorus, potassium, and lime effect on clover grown on a mine soil. *J. Environ. Qual.* **1994**, *23*, 759−765.

(22) Duncan, D. B. Multiple range and multiple F tests. *Biometrics* **1995**, *11*, 1−42.

(23) Fernie, A. R.; Willmitzer, L. Molecular and biochemical triggers of potato tuber development. *Plant Physiol.* **2001**, *127*, 1459−1465.

(24) Kelman, A.; McGuire, R. G.; Tzeng, K.-C. Reducing the severity of bacterial soft rot by increasing the concentration of calcium in potato tubers. *Soilborne Plant Pathogens: Management of Diseases with Macro- and Microelements*; Engelhard, A. W., Ed.; APS Press: Bethesda, 1989; pp 102−123.

(25) Marschner, H. Mineral Nutrition of Plants, 2nd ed.; Academic Press: Boston, 1995.

(26) Pittman, J. K.; Hirschi, K. D. Don't shoot the (second) messenger: endomembrane transporters and binding proteins modulate cytosolic Ca^{2+} levels. *Curr. Opin. Plant Biol.* **2003**, *6*, 257−262.

(27) Quintana, J. M.; Harrison, H. C.; Nienhuis, J.; Palta, J. P.; Kmiecik, K. Differences in pod calcium concentration for eight snap bean and dry bean cultivars. *HortScience* **1999**, *34*, 932−934.

(28) Quintana, J. M.; Harrison, H. C.; Palta, J. P.; Nienhuis, J.; Kmiecik, K. Calcium fertilizers fail to affect pod calcium concentration and yield of four snap bean cultivars. *HortScience* **1999**, *34*, 646−647.

(29) Quintana, J. M.; Harrison, H. C.; Palta, J. P.; Nienhuis, J.; Kmiecik, K.; Miglioranza, E. Xylem flow rate differences are associated with genetic variation in snap bean pod calcium concentration. *J. Am. Soc. Hortic. Sci.* **1999**, *124*, 488−191.

(30) McDole, R. E.; Westermann, D. T.; Kleinschmidt, G. D.; Kleinkopf, G. E.; Ojala, J. C. Idaho Fertilizer Guide: Potatoes. *Current Information Series No. 261*; University of Idaho, College of Agriculture.

(31) Palta, J. P. Role of Calcium in Plant Responses to Stresses: Linking Basic Research to the Solution of Practical Problems. *HortScience* **1996**, *31*, 51−57.

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